

A MEMBRANE POTENTIAL-MODULATED PATHWAY FOR Ca^{2+} EFFLUX IN RAT LIVER MITOCHONDRIA

Paolo BERNARDI and Giovanni Felice AZZONE

*CNR Unit for the Study of Physiology of Mitochondria and Institute of General Pathology, Via Loredan 16,
35100 Padova, Italy*

Received 1 December 1981

1. Introduction

Ca^{2+} transport is a basic mitochondrial function [1] and the inside-negative membrane potential ($\Delta\psi$) constitutes the driving force for Ca^{2+} influx on a native Ca^{2+} carrier, denoted as the Ca^{2+} uniporter [2,3].

Ca^{2+} distribution in vivo cannot attain electrochemical equilibrium. In fact a $\Delta\psi$ of ~ 180 mV at static head implies a Ca^{2+} accumulation ratio of 10^6 at equilibrium. Since cytosolic free Ca^{2+} is estimated to be $0.1\text{--}1\text{ }\mu\text{M}$ [4], intramitochondrial Ca^{2+} would be $0.1\text{--}1\text{ M}$, which is highly unlikely [5]. Also mitochondrial Ca^{2+} distribution does not reach equilibrium in vitro at high $\Delta\psi$ [6–8]. Consequently, it has been proposed that mitochondria possess a Ca^{2+} efflux pathway in parallel with the influx pathway and that Ca^{2+} distribution reflects a kinetic steady state rather than an equilibrium state [5–9].

After an elegant study in highly purified inside-out submitochondrial particles, which indicated a P_i -dependent Ca^{2+} uptake [10], the view has emerged that P_i may play a key role in the physiological pathway of mitochondrial Ca^{2+} efflux. This paper reports a novel observation, namely the stimulation by P_i of an energy-dependent Ca^{2+} efflux which leads to a shift of the steady state extramitochondrial free Ca^{2+} towards lower pCa_0 values. The distribution of Ca^{2+} is correlated with the value of $\Delta\psi$ indicating that $\Delta\psi$

modulates the activity of the efflux pathway. Thus an increase of $\Delta\psi$ above 130 mV leads to increased activity of the efflux pathway, with correspondent increase of extramitochondrial free Ca^{2+} at steady state. Ca^{2+} distribution at high $\Delta\psi$ appears therefore to reflect a kinetic steady state where uptake via the electrical uniporter is counterbalanced by efflux via the P_i -stimulated, Mg^{2+} -dependent pathway modulated by $\Delta\psi$.

2. Materials and methods

Mitochondrial preparation and experimental procedures were exactly as in [11].

3. Results

Fig.1 shows the effect of P_i on steady state Ca^{2+} distribution in the presence of Mg^{2+} . In (A) addition of mitochondria to a medium containing $\sim 18\text{ }\mu\text{M}$ free Ca^{2+} initiated a process of Ca^{2+} uptake which reached a steady state in ~ 2 min. At this point, addition of 0.3 mM P_i initiated a process of Ca^{2+} efflux which led to a new steady state in ~ 2 min. Fig.1 B shows that when Ca^{2+} uptake was initiated by the addition of mitochondria to a medium containing 0.3 mM P_i , an identical steady state Ca^{2+} distribution was reached. Under the conditions of Fig.1 A or B addition of small ($2.5\text{ }\mu\text{M}$) Ca^{2+} or EGTA pulses did not alter the set point, in that Ca^{2+} uptake or Ca^{2+} efflux ensued leading to the same level of pCa_0 preceeding the addition of Ca^{2+} or EGTA (not shown). The P_i -stimulated Ca^{2+} efflux shown in fig.1 is dependent on the presence of Mg^{2+} (app. K_m 0.94 mM Mg^{2+}), and exhibits a V_{\max} of $2\text{--}2.5\text{ nmol Ca}^{2+} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$.

Abbreviations: EGTA, ethylene-bis(oxoethylenitrilo)tetraacetic acid; BSA, bovine serum albumin; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Mops, 4-morpholinepropane sulfonic acid; HEDTA, N' -(2-hydroxyethyl)ethylene-diamine- N,N,N' -triacetic acid; TPMP⁺, triphenylmethylphosphonium ion; FCCP, carbonylcyanide- p -trifluoromethoxyphenylhydrazine; pCa_0 , $-\log [\text{Ca}^{2+}]$ outside the mitochondrial compartment; $\Delta\psi$, membrane potential

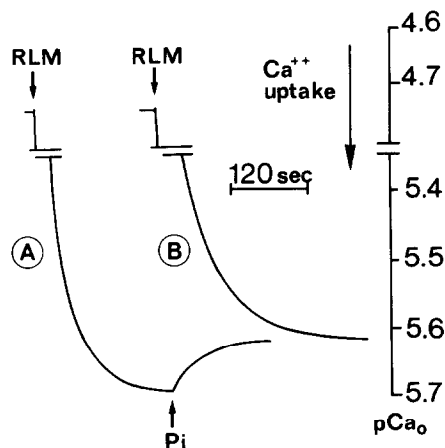


Fig.1. P_i -induced shift of steady state Ca^{2+} distribution. (A) The incubation medium contained 0.14 M sucrose, 40 mM choline, 10 mM Tris-Mops (pH 7.0), 2 mM $MgCl_2$, 0.5 mM β -hydroxybutyrate, 1 mg BSA/ml, 18 μ M Ca^{2+} . In (B) 0.3 mM P_i was added. Final vol. 5 ml; 30°C; when indicated 5 mg mitochondria (RLM) and 0.3 mM P_i were added.

Fig.2 shows a titration of the effect of P_i , with respect to the rate and extent of Ca^{2+} efflux. P_i at 10 μ M already caused a significant Ca^{2+} efflux, while the effect was maximal at ~ 0.2 –0.3 mM P_i . The lower trace shows that addition of hexokinase + glucose, by removing P_i , largely inhibited Ca^{2+} efflux with correspondent shift of the set point for external free Ca^{2+} at lower Ca^{2+} values.

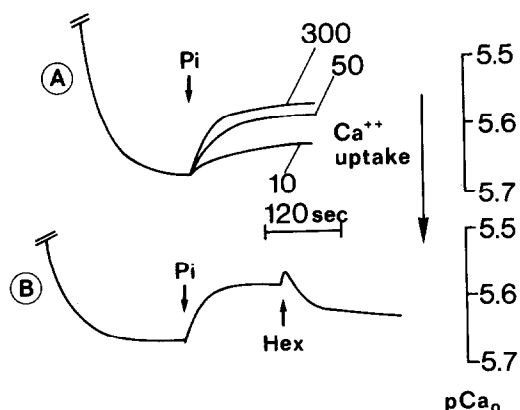


Fig.2. Dependence of Ca^{2+} efflux on $[P_i]$. Experimental conditions were as in fig.1(A) plus 5 mM succinate. The experiment was started by the addition of 5 mg mitochondria (not shown). Additions were: trace A, P_i as indicated by the numbers on each trace (nmol/mg protein); (B) 0.3 mM P_i and an excess hexokinase + 10 mM glucose (Hex). In (B) 10 μ M ATP was also present.

Parallel measurements of $\Delta\psi$ and ΔpH were performed under the conditions of these experiments. Before addition of P_i (trace 2A) $\Delta\psi$ was 168 mV and ΔpH was 52 mV. Upon addition of 0.3 mM P_i , ΔpH decreased to 43 mV with compensatory increase of $\Delta\psi$ to 177 mV. The P_i -stimulated, Mg^{2+} -dependent Ca^{2+} efflux occurs therefore against an increased $\Delta\psi$.

To analyze the relationship between P_i -stimulated Ca^{2+} efflux and $\Delta\psi$, we tested the effects of small concentrations of FCCP on steady state Ca^{2+} distribution. Fig.3 shows that, after P_i -stimulated Ca^{2+} efflux, addition of small amounts of FCCP caused increased Ca^{2+} uptake, until a critical concentration of uncoupler caused irreversible Ca^{2+} release. Since uncouplers are generally known to induce Ca^{2+} efflux, it was mandatory to exclude electrode artifacts. Control experiments were performed as follows. Mitochondria were incubated under the same conditions in the presence of 5 μ g antimycin A. Subsequently, EGTA was added to reach the same pCa_o maintained by respiring mitochondria in the presence of P_i . At this point addition of FCCP caused negligible effects on the pCa_o . Also no change in sensitivity of the assay was detected when the calibration of the electrode was performed with the Ca-HEDTA buffers, supplemented with 1 mg/ml BSA, in the absence or presence of the same concentrations of FCCP. In fig.3, due to the presence of BSA, a large discrepancy is established between BSA-bound and free FCCP. A titration of the FCCP-induced respiratory stimulation in the absence or presence of 1 mg BSA/ml indicated that the concentration of free FCCP in the presence of BSA is ~ 10 –20% of the total.

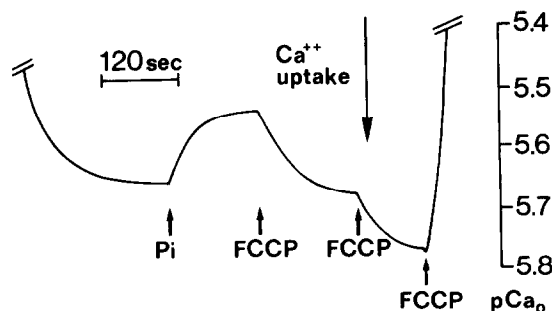


Fig.3. Effect of FCCP on steady state Ca^{2+} distribution. Experimental conditions were as in fig.1(A) plus 5 mM succinate. The experiment was started by the addition of 5 mg mitochondria (not shown). When indicated 0.3 mM P_i and 160 nM (first addition) or 200 nM (further additions) FCCP.

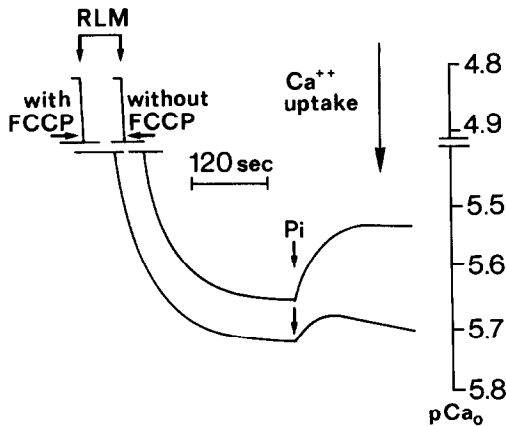


Fig.4. Inhibitory effect of FCCP on P_i -stimulated Ca^{2+} efflux. Experimental conditions were as in fig.1(A) plus 5 mM succinate. FCCP was 160 nM (left). When indicated 5 mg mitochondria (RLM) and 0.3 mM P_i .

Results similar in principle to those in fig.3 were obtained when $\Delta\psi$ was varied by addition of increasing K^+ concentration to valinomycin-treated mitochondria. Two Ca^{2+} -electrode traces are reported in fig.4, showing the Ca^{2+} steady state distribution in uncoupler-treated and untreated mitochondria and the subsequent phase of P_i -stimulated Ca^{2+} efflux. In the presence of small uncoupler concentrations the pCa_0 was higher. The uncoupler effect on Ca^{2+} distribution in the absence of exogenous P_i suggests that $\Delta\psi$ sustains a basal rate of Ca^{2+} efflux even in the absence of added P_i . Fig.4 also shows that the rate of P_i -stimulated Ca^{2+} efflux was considerably slower in uncoupler-treated mitochondria. This is in accord with an uncoupler-induced inhibition of the P_i -stimulated pathway for Ca^{2+} efflux.

Fig.5 analyzes the quantitative relationship between steady state pCa_0 and $\Delta\psi$, titrated with increasing concentrations of FCCP, in the presence of 0.3 mM P_i . pCa_0 was determined with the Ca^{2+} electrode, and $\Delta\psi$ was determined on $[^{14}C]$ TPMP $^+$ distribution on parallel samples. Before addition of P_i , pCa_0 was 5.71 and $\Delta\psi$ 165.5 mV (not shown). From these data it is apparent that above the critical value of ~ 130 mV and in the presence of P_i , Ca^{2+} distribution is dependent on the magnitude of $\Delta\psi$, in that an increase of the latter leads to stimulation of a mechanism for Ca^{2+} efflux with decrease of the steady state pCa_0 .

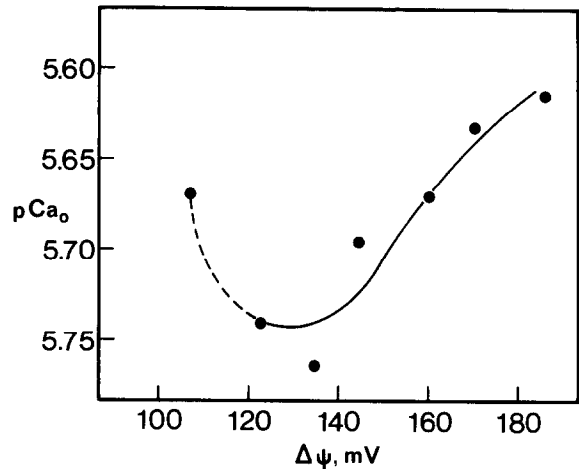


Fig.5. Relationship between steady state pCa_0 and membrane potential. The incubation medium was the same as fig.1(A) plus 5 mM succinate. Steady state pCa_0 values were determined with the Ca^{2+} -selective electrode, at $30^\circ C$. The experiments were started by the addition of 1 mg mitochondria/ml. After 5 min, 0.3 mM P_i was added. After attainment of steady state conditions, 40–400 nM FCCP was added. pCa_0 values refer to the steady state following addition of FCCP. Membrane potential was determined on parallel samples containing 0.04 μCi $[^{14}C]$ TPMP $^+$ (final vol. 2 ml, at $30^\circ C$). The experiments were carried out exactly as described for the determinations of pCa_0 and $\Delta\psi$ values refer to the steady state following addition of FCCP. The samples were vigorously vortexed to avoid anaerobiosis. Each value of $\Delta\psi$ is average of 2 independent expt. The dashed line is to indicate that below a membrane potential of ~ 125 mV true steady state conditions of Ca^{2+} distribution were not attained, and net Ca^{2+} efflux ensued shortly after the addition of FCCP.

4. Discussion

Operation of a Ca^{2+} efflux pathway in parallel with an influx pathway may result in a very slow Ca^{2+} cycling and then in a negligible energy drain. This is due to the fact that attainment of the kinetic steady state only requires that the rate of Ca^{2+} efflux through the independent pathway be equal to the rate of Ca^{2+} influx through the uniport. Mg^{2+} profoundly affects the kinetics of Ca^{2+} uptake, increasing the sigmoidicity of the relation between initial rate of Ca^{2+} uptake and external free Ca^{2+} concentration [12,13]. Then, the rate of Ca^{2+} efflux required to balance out the rate of Ca^{2+} influx falls below 5 nmol Ca^{2+} · mg protein $^{-1}$ · min $^{-1}$ [7,8,14]. This is perfectly compatible with the rate of the energy-dependent, P_i -stimulated Ca^{2+} efflux described in here. The energy demand for the

subsequent Ca^{2+} cycling is below 1 natom oxygen . $\text{mg protein}^{-1} \cdot \text{min}^{-1}$.

One of the most striking features of the Ca^{2+} efflux pathway described here is its dependence on $\Delta\psi$. These findings contrast with the observation that the steady state pCa_0 maintained by mitochondria is unaffected by the magnitude of $\Delta\psi$ at ≥ 125 mV [8]. However, these experiments were done in the absence of Mg^{2+} and P_i [8] both of which are required to stimulate the pathway for Ca^{2+} efflux described here.

The FCCP-induced Ca^{2+} uptake shown in fig.3 is in contrast with observations where uncouplers, even at the lowest effective concentration always caused Ca^{2+} efflux [15]. The contrast is however only apparent when it is considered that Ca^{2+} distribution may be determined by two mechanisms, either thermodynamic or kinetic, where the former and the latter predominate at $\Delta\psi$ values below and above 130–140 mV, respectively [8]. The FCCP-induced Ca^{2+} efflux was in fact observed [15] at a high Ca^{2+} load and either in the absence of weak acid (high ΔpH and low $\Delta\psi$) or in the presence of 5 mM acetate, and 5 mM K^+ + valinomycin (measured $\Delta\psi$ 84 mV). In both cases, as we have also found, FCCP depresses $\Delta\psi$ and causes Ca^{2+} efflux via reversal of the uniporter.

Although neither the mechanism of regulation of the Ca^{2+} efflux pathway by $\Delta\psi$ nor the thermodynamic force driving Ca^{2+} efflux are yet known, two possibilities exist:

(i) $\Delta\psi$ regulates the pathway directly, i.e., $\Delta\psi$ itself is the driving force for the P_i -stimulated Ca^{2+} efflux;
 (ii) $\Delta\psi$ regulates the pathway indirectly, and the driving force is different from $\Delta\psi$, for example a gated $\text{H}^+/\text{Ca}^{2+}$ exchange triggered by a $\Delta\psi$ -dependent conformational change. Such changes are known to occur in energy-transducing membranes [16–19].

Further work is needed to clarify the molecular nature of the pathway for Ca^{2+} efflux. The $\Delta\psi$ modulation appears however of paramount significance in vivo, in order to avoid low cytosolic and high matrix $[\text{Ca}^{2+}]$, as would be attained by a uniport carrier bringing Ca^{2+} at electrochemical equilibrium with a $\Delta\psi$ of 180 mV.

The high $\Delta\psi$ of resting mitochondria may result in activation of Ca^{2+} efflux, while the lower $\Delta\psi$ of phosphorylating mitochondria may result in inhibition of the efflux pathway. Experiments to be reported fully elsewhere indicate that this is exactly the case, and that cycles of ATP synthesis are paralleled by

cycles of Ca^{2+} influx and efflux, with an increase of matrix $[\text{Ca}^{2+}]$ during accelerated turnover of the Krebs cycle due to oxidative phosphorylation.

Acknowledgements

We thank Dr Tullio Pozzan for helpful discussions and for reading the manuscript. The expert technical assistance of Mr Paolo Veronese in the isotope experiments and the excellent typewriting of Mrs Franca Schiavon Mazzari are gratefully acknowledged.

References

- [1] Saris, N. E. and Åckerman, K. E. O. (1980) *Curr. Top. Bioenerg.* 10, 103–179.
- [2] Scarpa, A. and Azzone, G. F. (1970) *Eur. J. Biochem.* 12, 328–335.
- [3] Selwyn, M. J., Dawson, A. P. and Dunnet, S. J. (1970) *FEBS Lett.* 10, 1–5.
- [4] Williamson, J. R. and Cooper, R. M. (1980) *FEBS Lett.* 117 suppl., K73–85.
- [5] Azzone, G. F., Bragadin, M., Dell'Antone, P. and Pozzan, T. (1975) in: *Electron Transfer Chain and Oxidative Phosphorylation* (Quagliariello, E. et al. eds) pp. 423–429, Elsevier Biomedical, Amsterdam.
- [6] Puskin, J. S., Gunter, T. E., Gunter, K. K. and Russel, P. R. (1976) *Biochemistry* 15, 3834–3842.
- [7] Pozzan, T., Bragadin, M. and Azzone, G. F. (1977) *Biochemistry* 16, 5618–5624.
- [8] Nicholls, D. G. (1978) *Biochem. J.* 176, 463–474.
- [9] Carafoli, E. (1979) *FEBS Lett.* 104, 1–5.
- [10] Wehrle, J. P. and Pedersen, P. L. (1979) *J. Biol. Chem.* 254, 7269–7275.
- [11] Bernardi, P. and Pietrobon, D. (1982) *FEBS Lett.* 139, 9–12.
- [12] Vinogradov, A. and Scarpa, A. (1973) *J. Biol. Chem.* 248, 5527–5531.
- [13] Bragadin, M., Pozzan, T. and Azzone, G. F. (1979) *Biochemistry* 18, 5972–5978.
- [14] Nicholls, D. G. and Crompton, M. (1980) *FEBS Lett.* 111, 261–268.
- [15] Rottenberg, H. and Scarpa, A. (1974) *Biochemistry* 13, 4811–4817.
- [16] Ryrle, I. J. and Jagendorf, A. T. (1972) *J. Biol. Chem.* 247, 4453–4459.
- [17] McCarty, R. E. and Fagan, J. (1973) *Biochemistry* 12, 1503–1507.
- [18] Kraajenhof, R. and Slater, E. C. (1975) in: *Proc. 3rd Intl. Congr. Photosynthesis, Rehovot, Israel* (Avron, M. ed) pp. 955–996, Elsevier Biomedical, Amsterdam.
- [19] Gräber, P., Schlöder, E. and Witt, H. T. (1977) *Biochim. Biophys. Acta* 461, 426–440.